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Effects of rumen undegradable protein on intake, digestibility and rumen kinetics and fermentation characteristics of dairy heifers

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ABSTRACT

The aim of this work was to evaluate the influence of increasing amounts of rumen undegradable protein (RUP) on intake, nutrient digestibility, rumen kinetics and fermentation characteristics and N use efficiency of dairy Holstein heifers. Eight rumen-cannulated Holstein heifers (initial body weight 276 ± 8.3 kg) were used in a replicate 4 × 4 Latin square design with four dietary RUP amounts in the total dietary protein, as follows: 38% of RUP (38RUP), 44% of RUP (44RUP), 51% of RUP (51RUP) and 57% of RUP (57RUP). The experimental period was 84 days, subdivided into four periods of 21 days (14 days for adaptation + 7 days for collections). Eight spot collections of faeces, urine, ruminal content and omasal digesta were performed at 9-h intervals, as follows: on 15th day samples were collected at 0600 h and 1500 h; on 16th day samples were collected at 0000 h, 0900 h and 1800 h; on 17th day samples were collected at 0300 h, 1200 h and 2100 h. Two complete rumen evacuations were performed: one on 19th day 4 h after morning feeding and another on 21st day immediately before morning feeding. Total (kg/day) and relative (g/kg of body weight) intake of dry matter, crude protein (CP), neutral detergent fibre corrected for ash and protein were not affected ($P > 0.10$) by dietary RUP amounts. Ruminal ($P = 0.06$) and intestinal ($P = 0.09$) digestibilities of CP presented a tendency to decrease with increasing RUP. There were no effects of dietary RUP amounts ($P > 0.10$) on the rates of ingestion, passage and digestion (%/h) as well on the total volatile fatty acid concentration in the rumen. Urinary N excretion presented a tendency ($P = 0.08$) to decrease with increasing RUP. In addition, retained N presented a tendency ($P = 0.09$) to increase according to dietary RUP amounts and greater values were observed for treatments 51RUP and 57RUP. Microbial protein synthesis, microbial efficiency and the efficiency of use of N for microbial synthesis decreased with increasing RUP ($P < 0.05$). Metabolisable protein flow presented a tendency to increase ($P = 0.091$) and

Abbreviations: AN_{RO}, rumen outflow of ammonia nitrogen; CP, crude protein; CPI, crude protein intake; CPI/DOMI, ratio between CP intake and digestible organic matter intake; CP_{RO}, rumen outflow of crude protein; DM, dry matter; DOM, digestible organic matter; EMS, efficiency of microbial protein synthesis; iNDF, indigestible neutral detergent fibre; k_d, rate of digestion; k_i, rate of intake; k_p, rate of passage; MP, metabolizable protein; MP_{mic}, metabolisable protein from microorganisms; NDFap, neutral detergent fibre corrected for ash and protein contents; N, nitrogen; N_{end}, theoretical rumen outflow of endogenous N; OM, organic matter; pdNDFap, potential degradable NDFap; Pmic, microbial protein; RAN, rumen ammonia nitrogen; RDP, rumen degradable protein; RUP, rumen undegradable protein; VFA, volatile fatty acids; 38RUP, 38% of RUP in dietary CP; 44RUP, 44% of RUP in dietary CP; 51RUP, 51% of RUP in dietary CP; 57RUP, 57% of RUP in dietary CP

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greater values were observed for 51RUP and 57RUP. In general, urinary N excretion decreased, while the flow of metabolisable protein and RUP increased with increasing RUP levels. As a consequence of this pattern, the treatments 51RUP and 57RUP presented increases in the retained N.

1. Introduction

Microbial protein (Pmic) is the main source of amino acids for ruminants, mainly because of its amino acid profile, which is similar to that of milk and muscles. Despite the use of endogenous and recycled nitrogen (N) for Pmic synthesis, the rumen degradable protein (RDP) represents the most important source of N for Pmic synthesis, with a significant importance in the metabolisable protein (MP) flow (Ma et al., 2010; Mikolayunas et al., 2011).

However, once the microbial N requirements are met through RDP, the supply of rumen undegradable protein (RUP) may increase the MP flow and, consequently, improve animal performance and nitrogen (N) retention (Mezzomo et al., 2011; Tomlinson et al., 1997). Besides the increase in MP flow, the supply of RUP also may cause changes in ruminal metabolism, with alterations in Pmic synthesis, N availability, fibre degradation and N recycling (Batista et al., 2016; Reynal et al., 2005).

The effects of RUP supplementation on rumen kinetics, protein availability and N use have been reported for growing beef cattle and lactating dairy cows (Batista et al., 2016; Ipharraguerre and Clark, 2005; Mezzomo et al., 2011; Rufino et al., 2016). However, studies on the influence of protein degradability on dairy heifer performance (Lascano et al., 2016), in particular, the effect of RUP supplementation on digestive parameters of Holstein dairy heifers fed diets with high proportions of forage, are scarce (Lascano and Heinrichs, 2009; Zanton et al., 2007).

We hypothesised that increasing RUP may alter rumen kinetics and fermentation characteristics as well as increase MP flow and improve the N balance of Holstein dairy heifers. Aligned to the current need of information described above, we designed this study to evaluate the influence of increasing dietary RUP amounts on intake, total and partial digestibility, rumen kinetics and characteristics, as well as N use of dairy Holstein heifers.

2. Material and methods

All animal handling, surgeries and procedures of this study were approved by the Ethics Commission on the Use of Farm Animals of Universidade Federal de Viçosa (Viçosa, MG, Brazil), under protocol no. 039/2015.

2.1. Animals, treatments and management

Eight rumen-cannulated Holstein heifers, with an average initial BW of 276 ± 8.3 kg, were used in a replicate 4 × 4 Latin square design with four dietary RUP amounts in the total dietary protein, as follows: 38% of RUP (38RUP), 44% of RUP (44RUP), 51% of RUP (51RUP) and 57% of RUP (57RUP). The experiment started 40 days after the surgical procedure, when all animals had

Table 1
Ingredients and chemical composition of diets for dairy heifers fed different amounts of rumen undegradable protein (38, 44, 51 and 57% of dietary protein).

Item	Treatment			
	38RUP	44RUP	51RUP	57RUP
Ingredients, g/kg of DM				
Corn silage	700	700	700	700
Corn meal	110	110	110	110
Soybean meal	147	97	50	0
By-pass soybean meal[c]	0	50	97	147
Wheat bran	32	32	32	32
Urea	3	3	3	3
Minerals	8	8	8	8
Chemical composition				
Crude protein, g/kg of DM	151.4	152.0	152.6	153.2
Organic matter, g/kg of DM	935.8	935.8	935.7	935.7
NDFap[a], g/kg of DM	398.3	399.4	400.3	401.4
RUP/CP[b], g/kg of CP	389.8	441.3	489.4	540.2

Amounts of RDP and RUP of feedstuffs were calculated using values obtained in the *in situ* trial, which is described in a companion paper (Silva et al., 2018).

[a] neutral detergent fibre corrected for ash and protein.

[b] amount of RUP at total CP.

[c] Soypass BR®, Cargill Animal Nutrition, Campinas, São Paulo, Brazil.

recovered.

The heifers were housed in individual stalls with an area of 10 m^2 and provided with bunkers and water bowls. Corn silage was used as forage source, with a forage: concentrate ratio of 70:30 on dry matter (DM) basis. The diets were formulated to target an average daily gain of 1.0 kg/d, according to the recommendations of the NRC (2001; Table 1 and Supplemental Table S1). Animals had *ad libitum* access to feed, which was supplied at 07:00 h and 15:00 h.

2.2. Experimental period, collections and sampling

The experimental period was 84 days, subdivided into four experimental periods of 21 days each, which were preceded by a 15-day pre-experimental period to adapt the animals to the diets, facilities and management. The first 14 days of each period were used to adapt to the treatment (Machado et al., 2016), while the last 7 days were used for sample collections.

From the 12th to the 17th day of each period, ruminal infusions of 5 g/day of the complex Co-EDTA (555 mg/d of Co) (Udén et al., 1980) were administered four times daily at 00:00 h, 06:00 h, 12:00 h and 18:00 h.

The feeds supplied from the 15th to the 17th day as well as orts obtained from the 16th to the 19th day were sampled, pooled by heifer and stored at -20 °C. On the same days, eight spot collections of faeces, urine, ruminal content and omasal digesta were performed, with 9-h intervals between each collection, as follows: on the 15th day, samples were collected at 06:00 h and 15:00 h; on the 16th day, samples were collected at 00:00 h, 09:00 h and 18:00 h; on the 17th day, samples were collected at 03:00 h, 12:00 h and 21:00 h. In summary, samples were taken every 9 h over 3 days, representing a 3-h interval in a 24-h day. This collection scheme was applied to obtain digesta that represent the mean composition of ruminal and omasal digesta over the course of 1 day (Allen and Linton, 2007).

At each collection time, approximately 200 g of faeces were sampled via manual collection directly from the rectum, pooled by heifer and stored at -20 °C. In addition, approximately 50 ml of urine were sampled by stimulated urination. Immediately after sampling, 10 ml of urine were diluted in 40 ml of sulfuric acid (0.036 *N*) and stored (−20 °C) to prevent purine derivate degradation (Valadares et al., 1999). At the end of each period, the diluted samples were pooled by heifer.

Ruminal content was collected manually, filtered through four layers of cheese cloth and used to evaluate rumen ammonia nitrogen (RAN) and volatile fatty acids (VFA). Thereafter, an aliquot of 40 ml of rumen fluid was fixed using 1 ml of sulfuric acid (500 mL/L; 18 *N*) and stored at −20 °C for further measurement of RAN. Another aliquot of 20 ml was fixed using 5 ml of meta-phosphoric acid (250 g/L), pooled by heifers and stored at −20 °C for further analysis of VFA.

Prior to rumen sampling, omasal digesta collection was performed as described by Huhtanen et al. (1997), following the adaptations described by Mariz et al. (2013). At each collection time, approximately 600 ml of omasal digesta were collected and immediately filtered using a 100-μm nylon filter with 44% open area (Sefar Nitex 100/44, Sefar, Thal, Switzerland) to generate particle and fluid phase samples. At the end of each period, particle and fluid samples were stored at −20 °C for further analysis.

A complete rumen evacuation was performed on the 19th day, 4 h after the morning feeding, and on the 21st day, immediately before the morning feeding. Subsequently to the total rumen evacuation, the digesta was weighed and filtered through cheesecloth to obtain solid and liquid phases, which were individually weighed, sampled and stored at −20 °C. Thereafter, the digesta was re-constituted and returned to the rumen of the respective animals.

2.3. Laboratory analysis and calculations

Samples of feeds, orts, faeces and rumen content (fluid and particulate phases) were oven-dried (55 °C), while omasal digesta samples (fluid and particle phases) were freeze-dried. All samples were ground to 2 mm and 1 mm in a knife mill (Detmann et al., 2012). Subsequently, rumen content and omasal digesta (fluid and particles) samples were pooled by heifer and experimental period.

Samples ground to 1 mm were used to analyse DM (AOAC International, 2005; method 934.01), crude protein (CP; AOAC International, 2005; method 990.13), ash (AOAC International, 2005; method 942.05), neutral detergent fibre corrected for ash and protein contents (NDFap; using a heat-stable α-amylase and omitting sodium sulfite; Detmann et al., 2012; methods INCT-CA F-002/1; N-004/1 and M-002/1). Omasal digesta samples were also analysed for cobalt via an atomic absorption spectrophotometer (Spctr-800; Varian spectrometer, Harbor City, CA). In addition, each sample ground to 2 mm was incubated in quadruplicate into the rumen of two cows for 240 h, using non-woven textile bags with an area of 25 cm^2 and a density of 100 g/m^2, to determine indigestible NDF (iNDF) (Valente et al., 2011). Potential degradable NDFap (pdNDFap) was calculated as the difference between NDFap and iNDF.

The DM faecal excretion was estimated using iNDF as internal marker. This marker was also used in combination with Co-EDTA in a system of double marker to estimate the rumen outflow of DM and nutrients. The iNDF was used as marker of the particle phase and Co-EDTA as marker of the liquid phase, and their concentrations in the different omasal digesta phases were used to calculate the reconstitution factors (France and Siddons, 1986; Rotta et al., 2014).

The urine samples were analysed for their N content (AOAC International, 2005; method 990.13) as well as uric acid and allantoin concentrations, according to Fujihara et al. (1987) and Chen and Gomes (1992), respectively. In addition, creatinine concentration was measured using the colorimetric endpoint method, applying picrate and acidifier (Labtest Diagnóstica S.A. Lagoa Santa, Minas Gerais, Brazil).

Total daily urinary excretion was estimated using the daily creatinine excretion, as proposed by Chizzotti et al. (2008) for Holstein heifers. Ruminal microbial CP synthesis was estimated as a function of absorbed purines, which was calculated from the sum of uric acid and allantoin excretions, using equations proposed by Prates et al. (2012) for Holstein heifers.

The RUP intake was determinedusing the following equation:

$$RUP = CP_{RO} - P_{mic} - (AN_{RO} \times 6.25) - (N_{end} \times 6.25) \quad (1)$$

where RUP = undegradable protein intake (kg/day); CP_{RO} = rumen outflow of crude protein (kg/day); P_{mic} = microbial protein synthesis (kg/day); AN_{RO} = rumen outflow of ammonia nitrogen (kg/day); N_{end} = theoretical rumen outflow of endogenous N (kg/day; 2.27 g/kg DMI, according to Sauvant and Nozière (2013)).

The rumen degradable protein intake was calculated as follows (Cantalapiedra-Hijar et al., 2015):

$$RDP = CPI - RUP - (N_{end} \times 6.25), \quad (2)$$

where RDP = rumen degradable protein intake (kg/day); CPI = crude protein intake (kg/day); RUP = rumen undegradable protein intake (kg/day); N_{end} = theoretical rumen outflow of endogenous N (kg/day; 2.27 g/kg DMI, according to Sauvant and Nozière (2013)).

The flow of metabolizable protein from microorganisms was calculated as follows:

$$MP_{mic} = Pmic \times 0.64, \quad (3)$$

where MP_{mic} = metabolisable protein from microorganisms (g/day); Pmic = microbial protein (g/day); 0.64 = conversion factor of microbial protein to true digestible microbial protein, which considers that microbial protein has 80% of true protein and 80% of digestibility (NRC, 2001).

To estimate the flow of metabolisable protein from feedstuffs, the actual RUP intake was multiplied by RUP digestibility, which was estimated considering the RUP digestibility of each feed, according to the NRC (2001) and their proportions in each diet. In addition, total daily intake of metabolisable protein was estimated by the sum of metabolisable protein from microorganisms and metabolisable protein from feedstuffs.

Rumen pool size was calculated as the average of the pool size obtained via rumen evacuation on days 19 and 21, and was used to determine the rates of ingestion, passage and digestion, according to Allen and Linton (2007). The rate of digestion (kd; %/h) of each fraction was calculated as the difference between the rates of intake (ki; %/h) and passage (kp; %/h); these rates were then calculated according to Eqs (4) and (5), respectively.

$$k_i = \left(\frac{Intake}{Rumen\ pool}\right) \times 100 \quad (4)$$

where k_i = rate of intake (%/h); intake and rumen pool are given in kg/h and kg, respectively.

$$k_p = \left(\frac{Rumen\ outflow}{Rumen\ pool}\right) \times 100 \quad (5)$$

where k_p = rate of passage (%/h); rumen outflow and rumen pool are given in kg/h and kg, respectively.

The RAN concentration of rumen samples was determined using the colorimetric method (Detmann et al., 2012, method INCT-CA N-006-1). In addition, the VFA concentration was determined using HPLC (Dionex Ultimate 3000, Dionex Corporation, Sunnyvale, CA, USA), following the technique described by Siegfried et al. (1984).

Table 2
Intake of dairy heifers fed diets with different amounts of rumen undegradable protein (38, 44, 51 and 57% of dietary protein).

Item[a]	Treatment[c]				SEM[b]	*P*-Value
	38RUP	44RUP	51RUP	57RUP		
Intake, kg/d						
DM	6.81	6.69	6.78	6.75	0.360	0.91
OM	6.33	6.22	6.30	6.27	0.338	0.92
CP	1.00	0.99	1.01	0.97	0.083	0.29
NDFap	2.54	2.52	2.56	2.55	0.171	0.98
pdNDFap	1.72	1.71	1.75	1.75	0.191	0.86
DOM	4.51	4.45	4.56	4.60	0.245	0.68
Intake, g/d						
RUP	349^{B}	376^{B}	421^{A}	460^{A}	34.7	0.046
RDP	639^{A}	605^{AB}	578^{B}	513^{C}	36.8	0.022
Intake, g/kg BW						
DM	22.83	23.03	22.65	23.24	2.016	0.51
NDFap	8.52	8.67	8.58	8.61	0.917	0.97

Different letters indicate differences among dietary RUP amounts by Fisher's LSD ($P < 0.05$).

[a] DM = dry matter; OM = organic matter; CP = crude protein; NDFap = neutral detergent fibre corrected for ash and protein; pdNDFap = potential digestible neutral detergent fibre corrected for ash and protein; DOM = digestible organic matter; RUP = rumen undegradable protein; RDP = rumen degradable protein.

[b] Standard error of mean.

[c] The RUP percentage of the total dietary protein estimated by in situ trial was 39, 44, 49, and 54% for treatments 38RUP, 44RUP, 51RUP, and 57RUP, respectively.

2.4. Statistical analysis

The data were analysed using the MIXED procedure of SAS (SAS Institute Inc, 2008), according to the following statistical model:

$$Y_{ijkl} = \mu + RUP_i + LS_j + (RUP \times LS)_{ij} + A_{(j)k} + P_{(j)l} + \varepsilon_{ijkl}$$

where Y_{ijkl} = dependent variable; μ = overall mean; RUP_i = fixed effect of dietary RUP amounts; LS_j = random effect of Latin square; $RUP \times LS_{ij}$ = random effect of interaction between dietary RUP amounts and Latin square; $A(j)_k$ = random effect of animal within Latin square; $P(j)_l$ = random effect of period within Latin square and ε_{ijkl} = random error.

The interaction between dietary RUP and Latin Square was not significant for all variables and was removed from the statistical model. The data from different times of RAN sampling were included as repeated measures in the experimental model, and the effects of time and interaction between RUP and time were evaluated. The variance components, compound symmetry, heterogeneous compound symmetry, heterogeneous first-order autoregressive and unstructured matrices of (co)variance were tested. The matrix selection was based on the Corrected Akaike Information Criterion, and the heterogeneous compound symmetry was chosen. For all analyses, multiple comparisons between treatments were performed using Fisher's LSD test; differences were declared when $P < 0.05$ and tendencies when $0.05 \leq P < 0.10$.

3. Results

3.1. Intake and total, ruminal and intestinal digestibility

Total intakes were not affected ($P > 0.05$) by dietary RUP and exhibited average values of 6.76 ± 0.360, 6.28 ± 0.338, 1.00 ± 0.083, 2.54 ± 0.171, 1.73 ± 0.191 and 4.53 ± 0.245 kg/day for DM, organic matter (OM), CP, NDFap, pdNDFap and digestible OM (DOM), respectively (Table 2). Additionally, there were no effects ($P > 0.05$) on the relative intake (g/kg of BW), with average values of 22.94 ± 2.016 and 8.60 ± 0.917 for DM and NDFap, respectively.

Total intake of RUP (kg/day) was affected ($P = 0.046$) by dietary RUP and exhibited greater values for treatments 51RUP and 57RUP (Table 2). The RDP intake (kg/day) was affected ($P = 0.022$) by dietary RUP and decreased as RUP increased. Additionally, it is important to highlight that the actual RUP intakes of 349, 376, 421 and 460 represent 35, 38, 42 and 47% for treatments 38RUP, 44RUP, 51RUP and 57RUP, respectively.

Apparent total tract digestibility was not affected ($P > 0.05$) by dietary RUP amounts and exhibited average values of 699 ± 9.5, 722 ± 15.3, 704 ± 6.7, 529 ± 14.5, 778 ± 18.6 g/kg for DM, OM, CP, NDFap and pdNDFap, respectively (Table 3). Apparent ruminal digestibility of DM, OM, NDFap and pdNDFap was not affected ($P > 0.05$) by dietary RUP amounts, with average values of

Table 3
Apparent total tract, ruminal and intestinal digestibility of nutrients in dairy heifers fed diets with different amounts of rumen undegradable protein (38, 44, 51 and 57% of dietary protein).

Item[a]	Treatment[c]				SEM[b]	*P*-Value
	38RUP	44RUP	51RUP	57RUP		
Apparent total tract digestibility, g/kg						
DM	698	699	696	701	9.5	0.21
OM	712	716	724	734	15.3	0.32
CP	700	718	697	703	6.7	0.20
NDFap	513	523	530	549	14.5	0.12
pdNDFap	762	773	777	801	18.6	0.27
Apparent ruminal digestibility, g/kg of intake						
DM	322	332	335	360	22.2	0.20
OM	414	393	409	424	11.9	0.10
CP[d]	96	92	55	53	11.7	0.06
NDFap	403	401	406	425	26.7	0.79
pdNDFap	608	573	609	632	31.6	0.40
Intestinal digestibility, g/kg of rumen outflow						
DM	553	549	542	544	14.6	0.86
OM	516	532	533	533	23.8	0.83
CP	679	683	668	649	8.8	0.09
NDFap	181	217	207	210	29.7	0.78
pdNDFap	386	433	427	442	51.7	0.86

[a] DM = dry matter; OM = organic matter; CP = crude protein; NDFap = neutral detergent fibre corrected for ash and protein; pdNDFap = potential digestible neutral detergent fibre corrected for ash and protein.
[b] Standard error of mean.
[c] The RUP percentage of the total dietary protein estimated by in situ trial was 39, 44, 49, and 54% for treatments 38RUP, 44RUP, 51RUP, and 57RUP, respectively.
[d] Represents the ruminal disappearance of nitrogen, lost as ammonia.

337 ± 22.2, 410 ± 11.9, 409 ± 26.7 and 606 ± 31.6 g/kg, respectively. However, apparent ruminal digestibility of CP presented a tendency ($P = 0.06$) to decrease with increasing RUP values. In addition, apparent intestinal digestibility of CP also exhibited a tendency ($P = 0.09$) to decrease when RUP increased. Apparent intestinal digestibilities of DM, OM, NDFap and pdNDFap were not affected ($P > 0.05$) by dietary RUP amounts, with average values of 547 ± 14.6, 529 ± 23.8, 204 ± 29.7 and 422 ± 51.7 g/kg, respectively.

3.2. Rumen kinetics and characteristics

Rumen pools were not affected ($P > 0.05$) by dietary RUP and exhibited average values of 4.0 ± 0.36, 2.5 ± 0.26, 1.4 ± 0.18 and 1.1 ± 0.11 kg for DM, NDFap, iNDF and pdNDFap, respectively (Table 4). In addition, relative rumen pools of iNDF and pdNDFap were not affected by dietary RUP, with average values of 4.5 ± 0.33 and 3.9 ± 0.37 g/kg BW, respectively.

There were no effects ($P > 0.05$) of dietary RUP amounts on ingestion rates of DM, NDFap, iNDF and pdNDFap, with average values of 7.2 ± 0.40, 4.3 ± 0.34, 2.6 ± 0.24 and 6.5 ± 0.67%/h, respectively (Table 4). The passage rate was also not affected by treatments and exhibited average values of 4.8 ± 0.36, 2.6 ± 0.26 and 2.6 ± 0.37%/h for DM, iNDF and pdNDFap, respectively. In addition, digestion rate was also not affected by dietary RUP amounts, presenting average values of 2.4 ± 0.18 and 4.0 ± 0.44%/h for DM and pdNDFap, respectively.

The treatment 38RUP presented a greater RAN concentration ($P = 0.003$) in comparison to other treatments (Table 5). Interaction effect between RUP and hour of sampling was not observed ($P = 0.56$) on RAN concentration. However, RAN concentration was affected by hour of sampling ($P = 0.001$), presenting peaks after morning (07:00 h) and afternoon (15:00 h) feedings, with the greatest value at 09:00 h, which was the first sampling time after the morning feed (Fig. 1).

There were no effects ($P = 0.34$; Table 5) of dietary RUP amounts on total VFA concentration, which averaged 68.1 ± 3.10 mmol/L. Dietary RUP amounts also did not affect ($P > 0.05$) the proportion of each VFA and the relationship between acetate and propionate, with an average value of 4.06 ± 0.121 ($P = 0.72$).

3.3. Nitrogen balance and microbial protein synthesis

Ruminal nitrogen balance was affected ($P = 0.029$) by dietary RUP amounts and presented lower values for treatments 51RUP and 57RUP (Table 6). The dietary RUP amounts did not affect ($P > 0.05$) N intake and faecal N excretion, with average values of 160 ± 13.3 and 47.0 ± 4.52 g/day, respectively. However, N urinary excretion exhibited a tendency ($P = 0.08$) to decrease when RUP increased. Thus, the retained N exhibited a tendency ($P = 0.09$) to increase according to the dietary RUP amounts, and 51RUP

Table 4
Rumen pool and rates of ingestion, passage and digestion of dairy heifers fed diets with different amounts of rumen undegradable protein (38, 44, 51 and 57% of dietary protein).

Item[a]	Treatment[c]				SEM[b]	*P*-Value
	38RUP	44RUP	51RUP	57RUP		
Rumen pool, kg						
DM	3.99	3.78	4.01	4.03	0.359	0.49
NDFap	2.54	2.36	2.58	2.53	0.265	0.36
iNDF	1.42	1.30	1.36	1.35	0.180	0.70
pdNDFap	1.12	1.05	1.23	1.18	0.107	0.22
Rumen pool, g/kg BW						
iNDF	4.76	4.40	4.48	4.51	0.328	0.76
pdNDFap	3.77	3.69	4.10	3.96	0.369	0.47
Ingestion rate, %/h						
DM	7.12	7.46	7.09	7.20	0.402	0.70
NDFap	4.19	4.50	4.15	4.47	0.336	0.36
iNDF	2.42	2.70	2.55	2.65	0.237	0.59
pdNDFap	6.60	6.86	5.96	6.71	0.667	0.38
Passage rate, %/h						
DM	4.83	4.97	4.71	4.63	0.358	0.67
iNDF	2.46	2.76	2.63	2.73	0.262	0.58
pdNDFap	2.64	2.83	2.34	2.52	0.375	0.63
Digestion rate, %/h						
DM	2.28	2.49	2.37	2.57	0.181	0.25
pdNDFap	3.96	4.03	3.62	4.19	0.445	0.36

[a] DM = dry matter; NDFap = neutral detergent fibre corrected for ash and protein; iNDF = indigestible neutral detergent fiber; pdNDFap = potential degradable neutral detergent fibre corrected for ash and protein.
[b] Standard error of mean.
[c] The RUP percentage of the total dietary protein estimated by in situ trial was 39, 44, 49, and 54% for treatments 38RUP, 44RUP, 51RUP, and 57RUP, respectively.

Table 5
Rumen ammonia nitrogen and volatile fatty acids concentrations of dairy heifers fed diets with different amounts of rumen undegradable protein (38, 44, 51 and 57% of dietary protein).

Item[a]	Treatment[c]				SEM[b]	*P*-Value
	38RUP	44RUP	51RUP	57RUP		
RAN, mg/dL	16.7[a]	14.1[b]	14.7[b]	13.5[b]	1.18	0.003
Total VFA, mmol/L	63.5	70.7	68.7	69.5	3.10	0.34
VFA, mol/100 mol						
Acetate	66.7	66.9	67.5	67.3	0.59	0.55
Propionate	16.8	16.6	16.4	16.6	0.36	0.81
Butyrate	9.8	9.9	9.6	10.0	0.31	0.21
Isobutyrate	1.1	1.0	1.1	1.0	0.07	0.90
Valerate	2.5	2.5	2.5	2.3	0.17	0.65
Isovalerate	3.0	3.1	3.2	2.7	0.20	0.34
A/P	4.01	4.05	4.13	4.06	0.121	0.72

Different letters indicate differences among dietary RUP amounts by Fisher's LSD ($P < 0.05$).
[a] RAN = rumen ammonia nitrogen; VFA = volatile fatty acids; A/P = relation between acetate and propionate.
[b] Standard error of mean.
[c] The RUP percentage of the total dietary protein estimated by in situ trial was 39, 44, 49, and 54% for treatments 38RUP, 44RUP, 51RUP, and 57RUP, respectively.

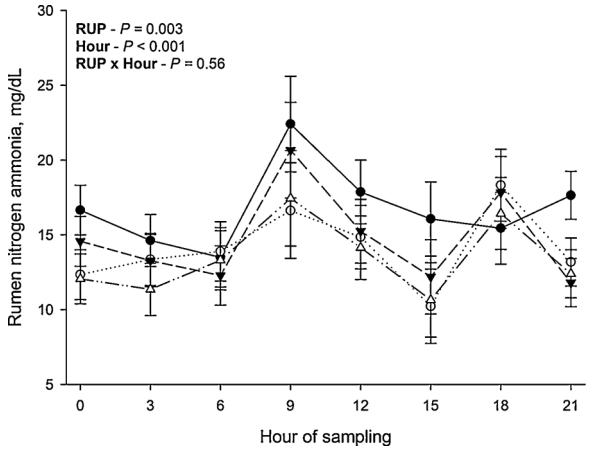

Fig. 1. Average rumen ammonia nitrogen concentration of Holstein heifers according to hour of sampling.

and 57RUP showed higher values than 44RUP and 38RUP. In addition, N use efficiency (N retained/ N intake) was affected by dietary RUP amounts ($P = 0.035$), with the highest values for the treatments 51RUP and 57RUP.

Dietary RUP affected Pmic synthesis ($P = 0.039$) as well as the efficiency of microbial protein synthesis (EMS; $P = 0.015$) and the N use efficiency for microbial synthesis ($P = 0.007$); all values decreased with increasing RUP (Table 6). In addition, the ratio between CP intake and digestible OM matter intake (CPI/DOMI) was lower ($P = 0.031$) for 57RUP when compared to the other treatments. Furthermore, dietary RUP affected ($P = 0.046$) the digestible RUP flow and presented a tendency ($P = 0.09$) to increase the MP flow, with greater values for 51RUP and 57RUP.

4. Discussion

The absence of any effects of RUP supply on voluntary feed intake (Bethard et al., 1997; Oliveira et al., 2008) as well as on apparent digestibility (Silva et al., 2018; Zanton et al., 2007) has already been observed in previous studies, corroborating the results of this study.

Ruminal CP metabolism involves the degradation of RDP, the recycling of N and Pmic synthesis, which occur simultaneously in the ruminal environment (Batista et al., 2016). Therefore, the apparent ruminal digestibility of CP does not represent the actual ruminal degradation of CP, but rather the ruminal disappearance of N (Arroyo et al., 2011; Rodrigues et al., 2017). In this context, the decrease of RDP intake (Table 2) was likely responsible for the tendency to decrease N lost as ammonia (Table 3) and for the decrease in RAN concentration (Table 5). The decrease in ruminal losses of ammonia (Table 3) was responsible for the tendency to decrease urinary N excretion (Table 6), as was observed by us in a companion paper (Silva et al., 2018) and by Batista et al. (2016). Consequently, there was an increase in N available for anabolism, represented by an increase in the retained N (Rufino et al., 2016;

Table 6
Nitrogen balance, microbial protein synthesis and protein flow of dairy heifers fed diets with different increasing amounts of rumen undegradable protein (38, 44, 51 and 57% of dietary protein).

Item[a]	Treatment[c]				SEM[b]	*P*-Value
	38RUP	44RUP	51RUP	57RUP		
Nitrogen balance						
Intake, g/d	160.0	158.4	161.6	155.7	13.28	0.29
RNB, g/d	15.2[a]	14.8[a]	8.8[b]	8.2[b]	2.47	0.029
Fecal, g/d	47.5	44.2	48.0	47.3	4.52	0.34
Urinary, g/d	97.3	97.4	89.7	85.5	6.13	0.08
Retained, g/d	15.2	16.8	23.9	22.9	7.54	0.09
NR/NI, g/g	0.10[b]	0.11[b]	0.15[a]	0.15[a]	0.046	0.035
Microbial synthesis						
Pmic, g/d	568[a]	541[ab]	519[bc]	477[c]	41.8	0.039
EMS, g/kg	126[a]	121[a]	114[ab]	104[b]	9.9	0.015
Nmic/NI, g/g	0.57[a]	0.55[ab]	0.51[bc]	0.49[c]	0.043	0.007
CPI/DOMI, g/kg	221.9[a]	222.3[a]	221.4[a]	210.8[b]	2.82	0.031
Protein flow, g/d						
RUPd	276[b]	301[b]	349[a]	381[a]	34.7	0.046
MP	639	647	682	687	14.5	0.09

Different letters indicate differences among dietary RUP amounts by Fisher's LSD ($P < 0.05$).

[a] RNB = ruminal nitrogen balance; NR/NI = relation between nitrogen retained and nitrogen intake; Pmic = microbial protein; EMS = efficiency of microbial protein synthesis (g of Pmic/kg of digestible organic matter intake); Nmic/NI = relation between microbial N synthesis and N intake; CPI/DOMI = relation between crude protein intake and digestible organic matter intake; RUPd = digestible rumen undegradable protein; MP = metabolizable protein.

[b] Standard error of mean.

[c] The RUP percentage of the total dietary protein estimated by in situ trial was 39, 44, 49, and 54% for treatments 38RUP, 44RUP, 51RUP, and 57RUP, respectively.

Table 6).

The tendency to decrease apparent intestinal digestibility of CP is probably linked to the proportion of Pmic and RUP that flowed to the small intestine (Table 6). The Pmic represented 62, 59, 55 and 51% of the total protein flowed to the small intestine for 38RUP, 44RUP, 51RUP and 57RUP, respectively. Although RUP and Pmic have previously been assumed to have a similar apparent intestinal protein digestibility (80%) (NRC, 2000), such equivalence was not observed in our study. As Pmic proportion decreased, apparent intestinal CP digestibility also decreased (Table 3), showing that the RUP used in this experiment, despite the good quality of the feedstuffs used, had a lower apparent intestinal digestibility than Pmic.

The observed decrease in Pmic synthesis as well as in EMS and efficiency of N use for microbial synthesis are usually consequences of decreased ruminal N and energy availabilities or a lack of synchrony between N and energy for microbial metabolism (Broderick et al., 2010; Cecava et al., 1990).

Apparently, ruminal N availability was not altered across the treatments, indicated by the positive values of ruminal CP degradability (Table 3), suggesting that all treatments had a positive rumen N balance (Table 6). Nevertheless, it must to be highlighted that RDP intake decreased when RUP supply increased (Table 2). Consequently, N recycling may have contributed to maintain the positive rumen N balance, since some authors have reported increases in rumen N recycling when RUP diets met 100% or even 150% of RUP requirements (Atkinson et al., 2007; Batista et al., 2016). On the other hand, Batista et al. (2017), in their meta analysis, have reported that for diets with around 15% CP (values similar to the diets of this study; Table 1), a decrease in the efficiency of the incorporation of recycled N into ruminal microbial N was observed, with an efficiency around 21%. This indicates that the efficiency of recycled N use is lower than the efficiency of consumed N use, which may be the responsible for the decrease in Pmic synthesis, EMS and N use efficiency for microbial synthesis. Additionally, the CPI/DOMI ratio, which represents the synchronism in the availability of energy and protein (Oliveira et al., 2016; Poppi and McLennan, 1995), was lower for 57RUP, which was the treatment with lower values of Pmic synthesis, EMS and N use efficiency for microbial synthesis (Table 6). A similar pattern has been observed by Oliveira et al. (2016) regarding Pmic synthesis.

Despite the decrease in Pmic synthesis and EMS (Table 6), ruminal fermentation was maintained under normal conditions, since there were no negative effects on VFA concentration (Table 5), DM and fibre digestibility (Table 3) and ruminal rates of fibre digestion and passage (Table 4). Thus, the inclusion of RUP had no deleterious effects on rumen dynamics, since the RAN concentration (Table 5; Fig. 1) was maintained at sufficient levels to support fibrolytic activity (7.1 mg/dL; Broderick et al., 2010) and close to the necessary level to maximise voluntary fibre intake (15 mg/dL; Detmann et al., 2009, 2014), even in treatments with greater RUP amounts. In addition, the availability of true rumen degradable protein, preferentially used by amylolytic bacteria, was not negatively affected by dietary RUP amounts, since the concentrations of isoacids and valerate remained unaltered across the treatments (Table 5; Lascano and Heinrichs, 2009).

The tendency to increase retained N, which was higher for treatments 51RUP and 57RUP (representing an actual RUP intake of 42 and 47% of total dietary protein, respectively), is a result of the increase in N available for deposition, since these treatments

presented a greater digestible RUP flow and the tendency for higher MP flow and lower urinary N excretion (Table 6; Tomlinson et al., 1997; Rufino et al., 2016). In a previous study, Bethard et al. (1997) have reported an increased feed efficiency of growing dairy heifers (animals with around 140 kg of initial BW) fed diets around 50% of RUP when compared to diets around 30% of RUP. In addition, Tomlinson et al. (1997) have reported a linear improvement in the ADG of growing dairy heifers (animals with around 220 kg of initial BW) when using 31, 43, 50 and 55% of RUP. Similarly, Ipharraguerre and Clark (2005), in a meta-analysis, have reported that highest milk yields of dairy cows were obtained using greater RUP levels.

Therefore, based on the various literature reports, our studies are generally in agreement with the results of previous similar studies. Furthermore, these results support the use of greater RUP amounts than those regularly recommended in the past years by the NRC (2001) – based on the beef cattle version (NRC, 2000) – which recommended an RUP value close to 30% to sustain an ADG of 1 kg/d. Moreover, new recommendations of beef NRC (2016) suggest an increment of up to 40% in the RUP requirements of beef cattle. Similarly, the necessity of increasing the RUP in diets for growing dairy heifers is known, but the lack of studies delays the establishment of higher RUP levels in the general guidelines.

5. Conclusions

The inclusion of RUP up to the level found in the treatment 57RUP neither affected voluntary feed intake nor digestion and passage rates of DM, CP and fibre, while intestinal digestibility of CP and N urinary excretion tended to decrease with increasing RUP levels. The MP flow and retained N tended to increase in the treatments 51RUP and 57RUP. Therefore, as the treatments 51RUP and 57RUP presented similar MP flow and N retention values, we recommend the use of treatment 51RUP for growing dairy heifers to achieve a weight gain of up to 1 kg/day.

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Conflict of interest

The authors have no conflicts of interest to declare.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.anifeedsci.2018.07.019.

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